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INFLAMMATORY-LIKE REACTION IN THE TUNIC OF *CIONA INTESTINALIS* (TUNICATA). I. ENCAPSULATION AND TISSUE INJURY

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ABSTRACT

Particulate (sheep erythrocytes, ascidian oocytes, stromata, colloidal carbon) or soluble agents (bovine serum albumin or hemoglobin, hemocyanin) were injected in varying doses into the tunic of *Ciona intestinalis*. This ascidian reacted by producing a capsule and/or tissue injury. Statistical analysis suggests that the two phenomena are independent, probably related to the nature and dose of the irritant.

Light histological observations showed granulocyte degranulation in the damaged tissue, suggesting that an acute inflammatory-like process is involved in the tunic reaction.

INTRODUCTION

To maintain body integrity, tunicates have evolved mechanisms which destroy and eject foreign materials. The defense responses include both humoral and cellular components. They could constitute a surveillance system ancestral to the vertebrate immune system (Parrinello and Patricolo, 1975, 1984; Parrinello *et al.*, 1977; Parrinello and Canicattì, 1982, 1983; Wright and Ermak, 1982).

Attempts to demonstrate immunological capabilities in the ascidian *Ciona intestinalis* have shown that it possesses natural (non-inducible) bacteriocidins (Johnson and Chapman, 1970) and hemagglutinins (Parrinello and Patricolo, 1975; Wright and Cooper, 1975) and that it reacts by phagocytosis and encapsulation to foreign materials inserted into the tunic (Parrinello *et al.*, 1977), and rejects a first set of tunic allografts (Reddy *et al.*, 1975). However the source of the natural defense responses needs further examination, while tunic graft rejection involves some persistent non-specific inflammatory responses.

C. intestinalis non-specifically reacts toward large concentrations of erythrocytes injected into the tunic and produces a capsule around them (Parrinello *et al.*, 1977). Cells infiltrate the area and release substances enveloping the foreign material. This response is often strong enough to produce large capsules visible through the tunic. Also in a variable number of treated specimens, the tunic matrix over the injected erythrocytes lysed, and a tunic wound was produced. Animals with the injured tunic survived for a period of time dependent on the seriousness of the trauma. In specimens which showed a slight reaction the wound healed. Preliminary light microscopic histological observations did not clarify the nature of such a reaction. Moreover, the data did not establish a relationship between the capsule and the injury.

In this study the *C. intestinalis* tunic injury and encapsulation produced by various doses of particulate or soluble agents were investigated by examining their external

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Abbreviations: SE = sheep erythrocytes; PBS = phosphate-buffered saline (0.01 *M* pH 7.4 phosphate buffer containing 0.15 *M* NaCl); BSA = bovine serum albumin; Hb = bovine hemoglobin; Hc = *Octopus vulgaris* hemocyanin.

appearance. It is shown that they are two un-related processes which most frequently appear as a result of high doses of either particulate material or soluble proteins. The capsule structure is described elsewhere (Parrinello and Patricolo, 1984). We now report some observations on the injured tissue. Histological studies relate the granulocyte degranulation to the injury process.

Materials and Methods

Adult *Ciona intestinalis* L. specimens (about 10–12 cm in length) were collected from the harbors of Palermo and Porticello, Italy. Animals showing a tunic free of external marine matter were selected; they were maintained in aerated and frequently renewed sea water at 15–18°C.

After several washings sheep erythrocytes (SE) (5×10^5, 5×10^6, 5×10^7 cells/ml) and colloidal carbon (G. Wagner, Lot C 11/1431 A) (2 and 20 mg/ml), were suspended in phosphate-buffered saline pH 7.4 (PBS). Hemoglobin-free red cell membranes were prepared using the method of Davis and Bakerman (1972); packed ghosts were suspended at final concentrations (v/v) of 1, 3, and 10% in PBS. The response to a more complex cellular system was investigated by injecting *C. intestinalis* oocytes collected from several specimens, washed and suspended in PBS ($1–1.2 \times 10^3$ oocytes/ml). Various concentrations (0.2, 2.0, and 20.0 mg/ml) of bovine serum albumin (BSA) (Sigma), bovine hemoglobin (Hb) (Sigma), and *Octopus vulgaris* hemocyanin (Hc) (kindly supplied by Dr. G. Nardi, Zoological Station, Naples), were prepared in PBS. 0.2 ml volumes of these preparations were injected into the tunic under the cuticle in the region of the sigmoid intestine with a syringe equipped with a 27-gauge needle.

Control specimens were injected with 0.2 ml PBS in each experiment.

The animals were inspected daily for signs of tissue reaction.

Statistical analyses were performed using analysis of variance; the acceptable level of significance was $P < 0.05$. For light optical studies a large fragment (about 1 cm wide) of the injured tunic was fixed in 70% ethanol and embedded in paraplast, 5 μm sections were stained with hematoxylin-eosin and Mallory's stains (Beccari and Mazzi, 1966).

Hemagglutinating activity was assayed as previously described (Parrinello and Patricolo, 1975). Blood was collected from the heart. Hemagglutination titers are expressed as reciprocal of the last dilution giving agglutination.

Results

The tunic reaction

Table I gives the external observations of the injected area during the tunic reaction. They show that, while PBS-injections never induce reaction in the control animals, particulate agents or soluble proteins can elicit two distinct types of tunic response. (1) SE, ascidian oocytes, BSA, Hb, and Hc induce a capsule which includes the injected materials. This appears 2 to 8 days after the injection as a whitish circular or elliptical disc (1.5–3.0 cm wide) included in the tunic tissue (Fig. 1). (2) SE, oocytes, the highest doses of stromata and colloidal carbon, BSA, and Hb induce a drastic response which produces a local injury of the tunic: a blister forms in the treated area, and the overlaying cuticle becomes thin and finally ruptures (Fig. 2a). When the foreign material is particulate the debris can disappear. In some specimens the two responses against SE, BSA, Hb, and Hc can occur together. In this case the injury can appear before or after the encapsulation. Both types of response show some degree

TABLE I

Reaction of Ciona intestinalis *to intratunic injection of particulate or soluble materials*

Irritant agent	Dose[1]	Injected specimens	Number of reacting specimens showing: Capsule A	Capsule plus injury B	Injury C	Days to produce 50% reacting specimens
SE	1×10^5	270	49 (18.1)	13 (4.8)	10 (3.7)	6–8
	1×10^6	422	176 (41.7)[a]	24 (5.7)	40 (9.5)	4–8
	1×10^7	937	332 (35.4)[b]	266 (28.4)[c]	33 (3.5)	3–6
Stromata from SE	1%	134	—	—	—	
	3%	111	—	—	—	
	10%	135	—	—	40 (29.6)	5
Oocytes	$1–1.2 \times 10^3$	50	36 (72.0)	—	12 (24.5)	5
Colloidal carbon	0.2 mg	113	—	—	—	
	2.0 mg	106	—	—	7 (6.6)	9
BSA	0.04 mg	100	1 (1.0)	19 (19.0)	—	8
	0.4 mg	100	16 (16.0)	40 (40.0)	—	4
	4.0 mg	100	—	8 (8.0)[c]	92 (92.0)	1
Hb	0.04 mg	130	4 (3.1)	—	—	4
	0.4 mg	124	48 (38.7)	15 (12.1)	2 (1.6)	2
	4.0 mg	126	14 (11.1)[d]	68 (53.9)[c]	14 (11.1)	2
Hc	0.2 mg	112	19 (16.9)	17 (15.2)	—	4
PBS	0.2 ml	627	—	—	—	

SE = sheep erythrocytes; BSA = bovine serum albumin; Hb = bovine hemoglobin; Hc = *Octopus vulgaris* hemocyanin; PBS = phosphate-buffered saline.

[1] In 0.2 ml. Percentage is reported in parentheses.

[a] $P < 0.01$ in comparison to B and C.

[b] $P < 0.05$ in comparison to C, $P < 0.01$ in comparison to C.

[c] $P < 0.01$ in comparison to C.

[d] $P < 0.01$ in comparison to B.

of modulation depending on the injurious agent. The capsule visible through the tunic appears in some of the specimens injected with erythrocytes (1×10^5–1×10^7), ascidian oocytes, BSA, Hb (0.04–4.0 mg), or Hc (0.4 mg). It is invisible when colloidal carbon or stromata is used even in high concentrations. The injury is produced by either particulate or soluble materials. Each agent, if injected in high doses, can elicit tunic damage as the only visible phenomenon (Table I).

The results in Table I suggest that encapsulation is not related to the injury formation. Significant differences ($P < 0.01$) were found between the frequencies of the capsule and injury responses produced by 1×10^6, 1×10^7 erythrocytes, or 4.0 mg BSA. Significant differences also resulted when "capsule plus injury" response was compared with each of the other two reactions produced when 1×10^6–1×10^7 SE, 4.0 mg BSA, or Hb were injected.

The dose of the irritant affects the response type and frequency, the largest dose being more effective in producing the tunic reaction. Different doses were examined statistically for each response type (Table II). Higher SE and protein concentrations

FIGURE 1. Capsule seven days after 0.4 mg bovine serum albumin injection into the tunic of *Ciona intestinalis* (arrowhead).

produced capsules in a significantly ($P < 0.01$) larger number of specimens. The highest doses also elicited an increased injury frequency.

There is variability in the time required for the appearance of the tunic reaction in about 50% of the reacting specimens in each treatment (Table I). The appearance time is inversely proportional to the dose and depends on the nature of the foreign material. Fast reactions (1–2 days) of either capsule, injury, or both were observed when concentrated protein solutions were used (4.0 mg BSA, 0.4–4.0 mg Hb) whereas slow reactions appearing in 4–8 days resulted at lower concentrations (0.04, 0.4 mg BSA, and 0.04 mg Hb). The same pattern characterized the effects of the various SE doses (see below) while only the highest concentration of stromata produced this response. The reaction time is also influenced by animal variability: in 10 different *C. intestinalis* groups (937 specimens) injected with the highest erythrocyte dose, about 50% of 631 specimens reacted within 3–6 days depending on each animal lot, the remainder reacted after 7–8 days. Variability in response frequency was also observed between the lots. The greatest variability characterized the mean frequency values of the injury responses. The mean values calculated from all experiments are indicated in Figure 3.

Hemagglutinating activity of the serum was tested with sheep or rabbit (RE) erythrocytes. To perform the assays the blood of five specimens was collected and pooled daily. Sera from PBS-injected animals showed no activity against SE, but agglutination titers of 2–4 were observed against RE. No changes were found in the hemagglutinating titers throughout the reaction period following SE injection.

The response toward particulate materials

Encapsulation is the most frequent response to erythrocytes and ascidian oocytes (Table I). Sheep erythrocytes can produce either a capsule or, less frequently, a tunic injury. Both responses are dose-dependent. The frequency of the tunic injury, even

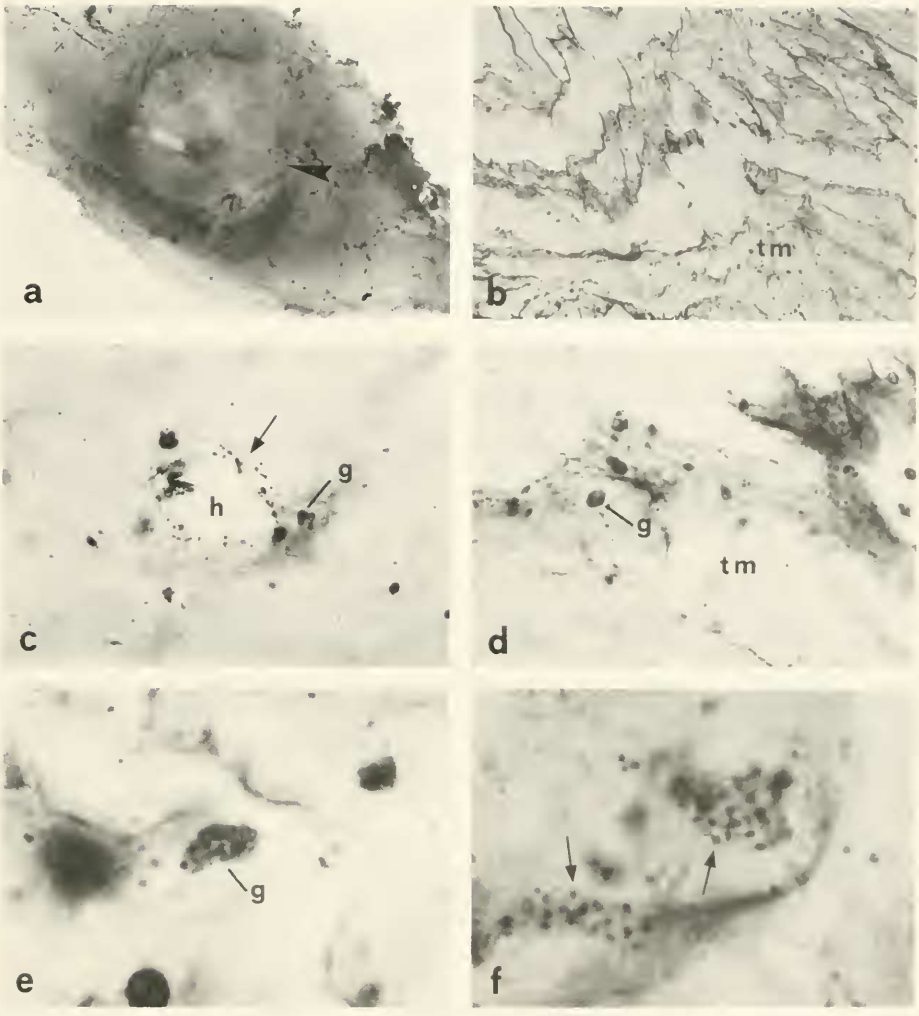

FIGURE 2. Tissue damage seven days after erythrocyte injection into the tunic of *Ciona intestinalis*. (a): Injured tunic (arrowhead); (b–f): transverse sections of the injured tunic (Mallory's stain); (b): lesion of the tunic, 80×; (c): hole in the tunic matrix (tm), 380×; (d): edge of the injured tunic, 380×; (e): eosinophil granulocyte, 1420×; (f): degranulation by cell membrane dissolution, 1420×. g = granulocyte; h = hole; arrows indicate granules.

when appearing with the capsule, increased with increased dosage (Fig. 3). The results in Figure 3 also show a pronounced variability in the responsiveness of the various animal groups to each dose.

The stromata at the highest dose and oocytes at the dose used also frequently elicited tunic injury, both being not very injurious agents. In fact, several days after the injection (5–8 days), these irritants produced tiny blisters (5–6 mm wide) containing gelatinous materials. Tissue damage rarely occurred when stromata were the irritants and the tunic subsequently healed.

Colloidal carbon was the least effective injurious agent even if injected in high quantities (2.0 mg/specimens). Only 6.6% of the 106 treated specimens showed a small blister like that described above.

TABLE II

Tunic reaction of Ciona intestinalis: *comparison of the dose response by analysis of variance*

Irritant agent	Dose compared	*P*-values[1]		
		Capsule	Capsule and injury	Injury
SE	1×10^5 vs. 1×10^6	$P < 0.01$	N.S.	$P < 0.01$
	1×10^5 vs. 1×10^7	$P < 0.01$	$P < 0.01$	$P < 0.01$
	1×10^6 vs. 1×10^7	$P < 0.01$	$P < 0.01$	$P < 0.01$
BSA	0.04 vs. 0.4 mg	$P < 0.01$	$P < 0.01$	—
	0.04 vs. 4.0 mg	N.S.	$P < 0.01$	$P < 0.01$
	0.4 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$
Hb	0.04 vs. 0.4 mg	$P < 0.01$	$P < 0.01$	N.S.
	0.04 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$
	0.4 vs. 4.0 mg	$P < 0.01$	$P < 0.01$	$P < 0.01$

SE = sheep erythrocytes; BSA = bovine serum albumin; Hb = bovine hemoglobin. N.S. = not significant.

[1] Data listed in Table I were examined.

The response toward soluble proteins

The responses vary with the different proteins and the various doses (Table I).

Bovine hemoglobin consistently elicited a capsule at low doses (0.04, 0.4 mg). Tunic injury became frequent, either alone (11.1%) or with capsule (53.9%), when 4.0 mg protein was injected.

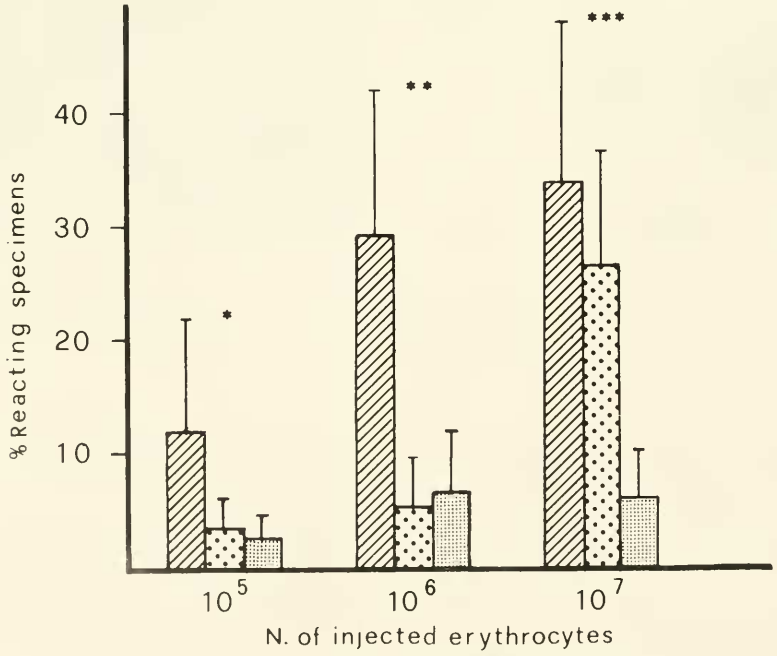

FIGURE 3. Diagram illustrating tunic reaction of *Ciona intestinalis* to increasing sheep erythrocyte doses; values are mean ± S.D., *n = 4, **n = 6, ***n = 10. ▨ = capsule, ⊡ = capsule plus injury, □ = injury.

Capsules were also produced by low doses (0.04, 0.4 mg) of bovine serum albumin but the tunic injury was the most evident response induced by this irritant. Tissue damage and a capsule were observed at 0.04 mg BSA in 19% of 100 specimens. This proportion increases to 40% at 0.4 mg. A dose of 4.0 mg BSA was consistently injurious. Injury appeared rapidly but capsules were not observed. In some specimens the injury was so severe as to produce serious tissue damage in a short time.

Hemocyanin was used in a single dose (0.4 mg) and it induced, from 4 to 7 days, tunic wounding and/or capsules in 32.1% of the 112 specimens.

Histological observations of the damaged tissue

To study the injured tunic, specimens which reacted against 1×10^7 SE or oocytes were fixed after 6 and 8 days, respectively. Additional specimens reacting in 3–4 days against 4.0 mg BSA or Hb were also prepared. Transverse and longitudinal sections, stained with Mallory's or hematoxylin-eosin stains, showed tunic lesions (Fig. 2b, c): the tunic matrix has flaked off revealing a large wound.

Granular amoebocytes (6.2–7.6 μm) are numerous and are readily identified by their eosinophil granules (0.6–0.8 μm) (Fig. 2d, e). Some of these cells can discharge their granules. This degranulation is particularly evident on the edges of the injury and can be related to the holes which occur in the tunic matrix (Fig. 2c). The degranulation mechanism can coincide with the loss of cell membrane integrity and the bulk leakage of all cell contents (Fig. 2f).

The wound produced in the tunic matrix by the injection contains particulate material. Cell infiltrate was at the wound edges. The cells appear to be granular amoebocytes which had discharged their granules. This process also occurs among the injected material masses and was particularly evident when oocytes were injected (Fig. 4). In some sections, eosinophil granulocytes are so numerous and the material in the wound so conspicuous that the deep staining masks the cells. The same features characterize the tunics injured by BSA or Hb.

To determine whether the injection procedure influenced the tunic reaction, specimens treated with PBS were studied after six days. Histological sections of the whole tunic from five specimens showed that granulocytes were rare in three specimens. In the others some of these cells were irregularly distributed near the epithelium.

Discussion

In vertebrates, inflammation is a response of living tissues to local injury. This process is characterized by local heat, swelling, redness, and pain, and leads to the accumulation of blood cells and fluid. It includes the removal of foreign materials and the disposal of damaged tissue followed by healing (Ryan and Majno, 1977). In invertebrates some features of vertebrate inflammation are not applicable. This reaction is a non-specific cellular response which includes phagocytosis, encapsulation, and wound healing (Metchnikoff, 1892). Inflammatory-like responses have been shown in annelids (Stein and Cooper, 1983), molluscs (*cfr.* Cheng, 1981), arthropods (Ratner and Vinsons, 1983), and echinoderms (*cfr.* Smith, 1981). In tunicates the encapsulation process can be provoked by natural invaders or experimentally inserted agents (*cfr.* Wright, 1981; Wright and Ermak, 1982; Wright and Cooper, 1983). The tunic reaction of *C. intestinalis* is an inflammatory-like response typical of invertebrates. Small quantities of erythrocytes injected into the tunic are cleared by phagocytosis, whereas encapsulation occurs when larger amounts are used (Wright and Cooper, 1975; Parrinello *et al.*, 1977). Increasing doses of sheep erythrocytes induce large capsules evident in the tunics of an increasing number of reacting specimens. The absence of tunic vessels probably allows for the lengthy persistence of the irritant promoting a

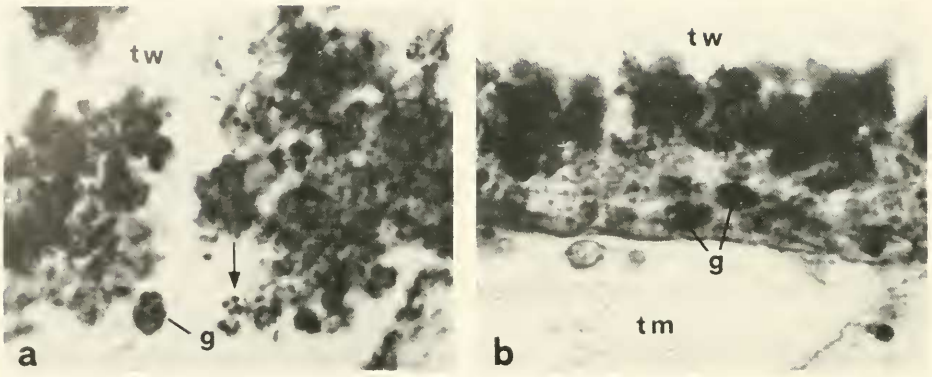

FIGURE 4. Tunic wound induced by ascidian oocyte injection. (a): Oocyte debris inside the wound, 850×; (b): granulocytes on the edge of the wound, 850×. g = granulocyte; tm = tunic matrix; tw = tunic wound; arrow indicates granules.

type of chronic inflammation. The results also suggest that encapsulation is not only elicited by particulate invaders of large size but soluble proteins can also induce capsules while large amounts of colloidal carbon or stromata fail to produce strong reactions.

The irritants that induce encapsulation can also cause tissue injury. The frequency and the seriousness of the damage, and the speed with which it occurs, are dependent on the dose and/or the nature of the irritant. Proteins seem to be more injurious agents and the highest dose of BSA induces such fast reactions (24–48 hours) that a capsule does not become visible. There is little response to stromata and colloidal carbon even in large doses.

The injury process is probably related to degranulation of the granulocytes and is not related to encapsulation. On the other hand, a capsule does not indicate a repairing process after the tissue injury. Statistically significant differences between some groups of results confirm these considerations.

Histological observation of wounds containing oocytes and SE remains suggest that the eosinophil granulocytes and the degranulation process may be involved in the removal of large degradable masses of inflammation-provoking agents. Tunic damage may be a lesser effect of a defense mechanism. It can depend on the irritant and/or dose, as shown by statistical analysis. Lysosomal mechanisms, like those proposed for neuthrophil involvement in the mammalian inflammatory reactions, could be responsible for the tunic injury (Hirschhorn, 1974; Gleisner, 1979). Agents could cause granulocyte disruption coincident with the loss of membrane integrity and possible release of lysosomal enzymes. We do not know if bacteria, either occurring naturally in the tunic (De Leo *et al.,* 1981) or resulting from contamination of the injection wound, may be involved in the tunic lysis. Moreover, cytoplasmic vacuoles of the *C. intestinalis* granulocytes can contain small deposits of vanadium associated with sulfur compounds (Rowley, 1982, 1983) which might, when released, participate in the inflammation process.

Even if the exact nature of the signal is unknown, it can be assumed that the concentration and activity of chemotactic substances could account for the strength and speed of the tunic reaction. Evidence concerning the mammalian inflammation process suggests that proteins can act directly or, after proteolysis, liberate chemotactic fragments (Houck *et al.,* 1971), while active factors may be produced by digestion of foreign material (*cfr.* Hirschhorn, 1974; Gleisner, 1979).

The contribution of naturally occurring humoral factors (bactericidins, hemagglutinins) in the non-self recognition mechanisms and their defensive role needs

further study (Wright and Ermak, 1982). The results reported in this paper suggest that hemagglutinins are not needed in the erythrocyte-induced inflammatory-like reactions, and that the inflammatory cells apparently recognize foreigness without the intervention of soluble factors.

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